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Comments on diagnosis of amoebic gill disease (AGD) in turbot, *Scophthalmus maximus*

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Abstract

Comments on diagnostic procedures and their pitfalls, which were experienced mainly during the research of AGD in turbot, *Scophthalmus maximus*, are presented. Diagnostically important data are summarised and the necessity of morphological recognition of the agent is highlighted.

Since the exact recognition of aetiology constitutes the basic prerequisite for the treatment, control and prevention of a disease problem in any type of aquaculture, the appropriate methodology for detection and identification of agents is of primary importance. Among the diagnostic methods recommended for the amoebic gill disease (AGD) affecting cultured Atlantic salmon, *Salmo salar*, wet mounts of gill tissue and mucus scraped from gills, air dried mucus smears stained with Quick Dip® or subjected to IFAT, and histological examination were reported by Kent *et al*. (1988), Noga (1996) and Zilberg *et al*. (2000).

Our observations may be of interest to those who are beginning the study of AGD. The data presented are based on our experience with AGD caused by *Paramoeba* / *Neoparamoeba* spp. in turbot, *Scophthalmus maximus*, and on a less extensive experience with AGD in European seabass *Dicentrarchus labrax*, sharpsnout seabream *Diplodus puntazzo* and Atlantic salmon *Salmo salar* (Dyková *et al*. 1995, 1998, 1999, in press). References are provided for additional reading.

Preliminary diagnosis of AGD

The examination of wet mounts of gill tissue and mucus scraped from gills was of diagnostic value in advanced stages of AGD, when gills of turbot were heavily infested with amoebae. Since all paramoebid trophozoites are polymorphic (Fig. 1), the recognition of the agent required some degree of experience. The shape of trophozoites that moved slowly in dense mucus resembled that observed in histological sections (Figs. 2,5,6). The cytoplasm of trophozoites was almost always highly vacuolated. In the mucus mixed with seawater, amoebae could be observed also as floating stages (Fig. 3). They could be concentrated by washing gill arches in seawater followed by low speed centrifugation. When a drop of the loose pellet obtained following centrifugation was placed on a coverslip into wet chamber for about 30 minutes, trophozoites attached to coverslip. Their morphology could then be observed in a hanging drop using depression slide (Fig. 1).

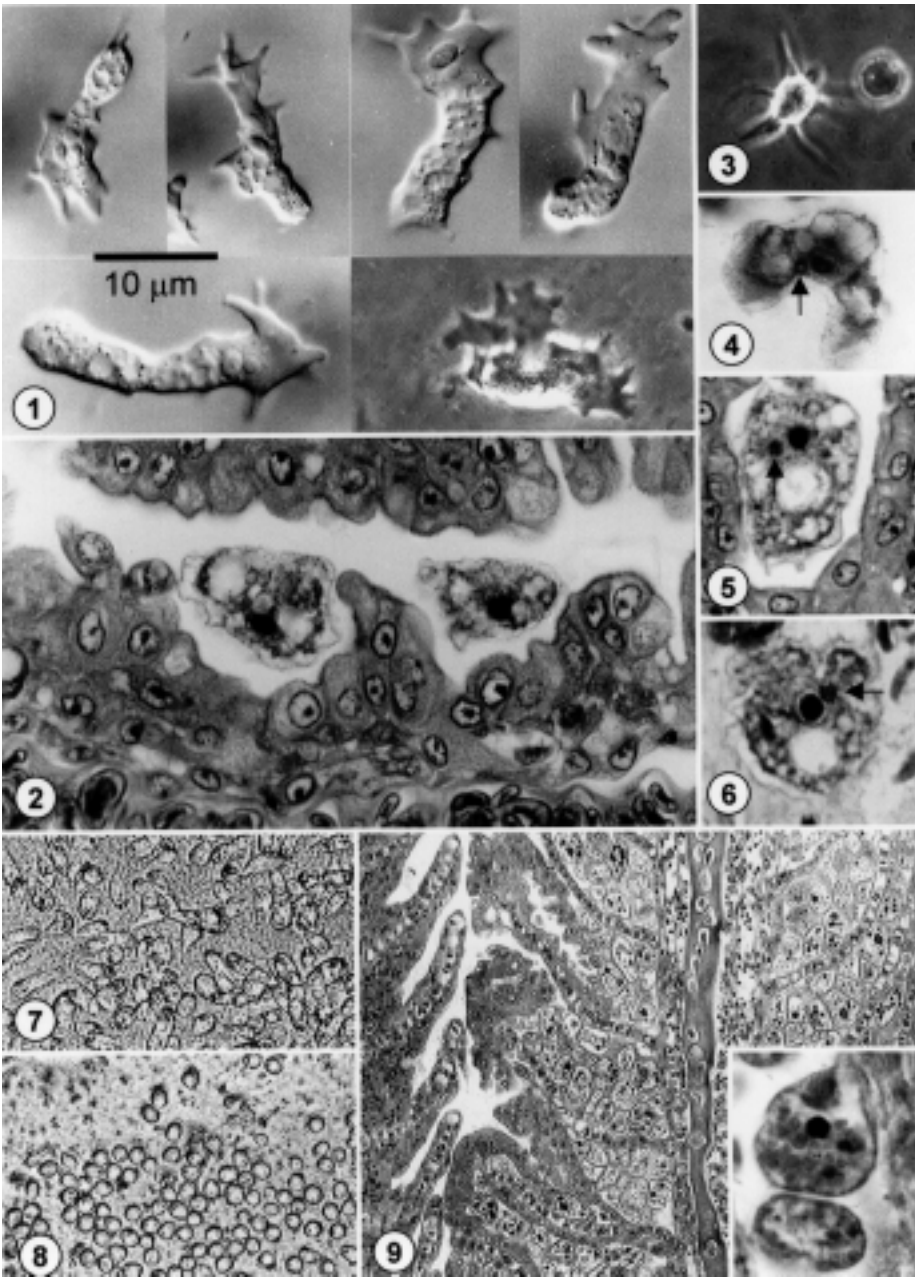

Fig. 1-6. Trophozoites of *Neoparamoeba* spp. Fig. 1. Trophozoites grown on agar plates as observed in hanging drop preparation and through culture flasc wall in liquid medium (bottom right). Bar = 10 µm. Fig. 2. Trophozoites at the surface of gill epithelium, x 1000. Fig. 3. Floating stage of *Neoparamoeba* sp. Fig. 4. Parasome, i.e., *Perkinsiella amoebae* (arrow) visualized by Feulgen reaction. Figs. 5 & 6 Trophozoites in histological sections stained with hematoxylin & eosin with parasomes (arrows). Figs. 7-9. Histophagous ciliates. Fig. 7. Trophozoites as seen on agar plate through petri dish. Fig. 8. Cysts of ciliates as seen on agar plate through petri dish. Fig. 9. Gills of *Scophthalmus maximus* heavily infected with histophagous ciliates, H & E, x 250. Inset – trophozoites in histological section, x 1000.

Stained preparations

In order to visualise the nucleus and the so-called parasome, which in fact is a symbiotic organism (*Perkinsiella amoebae*) known to occur in the cytoplasm of trophozoites of three genera of the family Paramoebidae (Dyková *et al*. in press), trophozoites were left to attach to slides and then stained with hematoxylin and eosin, Feulgen reaction, and DNA staining (Hoechst 33258). In all tested fixatives (Davidson's fixative, ethanol-sublimate and 95% ethanol respectively) applied immediately after seawater was removed the trophozoites attached to slides retained their characteristic shape (Fig.4). This was considered important in view of the fact that in air-dried preparations, whatever stain used, trophozoites lost their characteristic shape and size and were difficult to compare with live amoebae. When precleaned slides were used, the adhesion of trophozoites was improved and loss of organisms was minimal.

Final diagnosis of AGD

In turbot, the final diagnosis was based on series of histological examinations of gills in various phases of infection. Histological findings revealed the cause of lesions, verified the primary role of amoebae and explained the sequence of lesions (Dyková *et al*.1995, 1998). The symbiotic organism localised near the nucleus was the target detail that made possible the identification of amoebae in sections (Figs. 5, 6). Histology also proved that in the final phase of infection, when the typical gill structure was completely transformed, the agent was present not necessarily in huge numbers. The fixative of choice was Davidson's fixative, which rapidly penetrates and enables observation of amoebae attached to gill tissue in samples processed for histology.

Isolation, culture and subculture of amoebae

Repeated isolations of the agent from infected tissue supported the results of histopathological studies. In addition, isolation attempts revealed the presence of many other accompanying organisms in affected tissues (Dyková *et al*.1999), of which the most important were histophagous ciliates (Figs. 7 to 9) belonging most probably to Scuticociliatida. They were isolated along with the agent of AGD from gills of turbot (sampled in three consecutive years from farms in NW Spain), from gills of seabream *Diplodus puntazzo* and seabass *Dicentrarchus labrax* (from a Mediterranean farm) and also from gills of Atlantic salmon *Salmo salar* (from an Irish farm). In the latter fish species, *Paramoeba* infection was suspected but not confirmed; however, amoebae belonging to three other genera were isolated (unpublished data). Together with ciliates, flagellates multiplied in tissue samples as soon as their decay began, when placed on non-nutrient agar. Although ciliates and flagellates could be easily recognised because of their motility in fresh cultures, in older cultures they formed cysts, which could be confused with cysts of amoebae (Fig. 8). The ciliates could also be confused with amoebae in histological sections (Fig. 9). Isolation attempts ranging from clinically healthy specimens to those with slight behavioural alterations revealed that grossly visible lesions are not necessarily present when the agent has colonised gills and distributed throughout the whole turbot stock.

The methodology for isolation of this agent has been described several times (Page, 1967; De Jonckheere, 1980; Dyková *et al*., 1997). Isolations are inappropriate as a routine diagnostic tool, but are important in new outbreaks of AGD, in new fish host species affected and also in monitoring cage or tank hygiene. Live trophozoites can be observed in mucus long after the death of the fish. Isolation attempts showed that they can survive in decaying tissues for more than three days. Primary isolations can fulfill diagnostic requirements, while subculturing and cloning, which are time consuming and often very difficult, are required for detailed study of the agent.

Since many free-living organisms were found to colonise turbot gills together with primary agent of AGD (Dyková *et al*., 1999), diagnosis has to be primarily based on morphological recognition of the pathogen. Sampling for diagnostic purposes should follow the methods outlined in Austin and Austin (1989) which have generally been accepted for other infections. In addition to moribund and freshly dead fish, apparently healthy specimens with slight clinical symptoms (behavioural or colour changes) also have to be sampled. The discrepancies in presumptive diagnosis of AGD by gross gill checks have been mentioned by Zilberg *et al*. (1999) in Atlantic salmon. The same applies to turbot: in three consecutive samplings in various farms, grossly visible lesions were not always present after the agent had already colonised gills and spread throughout the whole stock. In order to recognise early that a stock is threatened with AGD, constant monitoring of *Paramoeba* /*Neoparamoeba* trophozoites is recommended in clinically healthy fish and those in suboptimal health condition. The indirect fluorescent antibody technique (IFAT), highly recommended for the detection of *Paramoeba* cells in Atlantic salmon (Zilberg *et al*. 1999), has not been used for diagnosis of AGD in turbot.

Repeated isolation of ciliates accompanying the primary agent of AGD along with the fact that severe infection of gills and other organs with histophagous ciliates has been described in turbot (Dyková & Figueras 1994) stress the importance of identification of histophagous ciliates (work in progress). Among the issues remaining to be resolved is the relationship of histophagous ciliates to lesions produced in AGD by *Paramoeba* /*Neoparamoeba* spp. and whether the pathogenic role of ciliates could predominate under certain conditions. This requires an improved understanding of relationship between both groups of organisms, which belong to potential pathogens capable of independent existence outside the fish.

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References

Austin, B., Austin, D.A. (Eds.) (1989) Methods for the microbiological examination of fish and shellfish. Ellis Horwood Ltd., England, 317pp

De Jonckheere, J.F., (1980) Growth characteristic, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* sp. Appl. Environ. Microbiol. 39, 681-685

Dyková, I., Figueras, A. (1994) Histopathological changes in turbot *Scophthalmus maximus* due to a histophagous ciliate. Dis. Aquat. Org. 18, 5-9

Dyková, I., Figueras, A., Novoa, B. (1995) Amoebic gill infection of turbot, *Scophthalmus maximus*. Folia Parasitol. 42, 91-96

Dyková, I., Machá_ková, B., Pecková, H. (1997) Amoebae isolated from organs of farmed tilapias, *Oreochromis niloticus*. Folia Parasitol. 44, 81-90

Dyková, I., Figueras, A., Novoa, B., Fernandez-Casal, J. (1998) *Paramoeba* sp., an agent of amoebic gill disease of turbot, *Scophthalmus maximus*. Dis. Aquat. Org. 33, 137-141

Dyková, I., Figueras, A., Novoa, B. (1999) Epizoic amoebae from the gills of turbot *Scophthalmus maximus*. Dis. Aquat. Org. 38, 33-38

Dyková, I., Figueras, A., Peric, Z. *Neoparamoeba* Page, 1987: Light and electron microscopic observations on six strains of different origin. (Accepted for publication in DAO)

Kent, M.L., Sawyer, T. K., Hedrick, R. P. (1998) *Paramoeba pemaquidensis* (Sarcomastigophora: Paramoebidae) infestation of the gills of coho salmon *Oncorhynchus kisutch* reared in seawater. Dis. Aquat. Org. 5, 163-169

Noga, E. J. (1996) Fish Disease: diagnosis and treatment. Mosby-Year Book, Inc., St. Louis, 367 pp

Page, F.C. (1967) Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. J. Protozool.14, 499-521

Zilberg, D., Nowak, B., Carson, J., Wagner, T. (1999) Simple gill smear staining for diagnosis of amoebic gill disease. Bull. Eur. Ass. Fish Pathol. 19, 186